REMARKS / ARGUMENTS

The Examiner has maintained that the claimed ratios would be inherent and/or obvious by routine optimization. Applicants submit for the Examiner's reconsideration the claims and arguments as presented previously, in light of the following summary of case law, from Section 2144.05 of the M.P.E.P. entitled "Obviousness of Ranges:"

> B. Only Result-Effective Variables Can Be Optimized
>
> A particular parameter must first be recognized as a result-effective variable, i.e., a variable which achieves a recognized result, before the determination of the optimum or workable ranges of said variable might be characterized as routine experimentation. *In re Antonie*, 559 F.2d 618, 195 USPQ 6 (CCPA 1977) (The claimed wastewater treatment device had a tank volume to contractor area of 0.12 gal./sq. ft. The prior art did not recognize that treatment capacity is a function of the tank volume to contractor ratio, and therefore the parameter optimized was not recognized in the art to be a result- effective variable.). See also *In re Boesch*, 617 F.2d 272, 205 USPQ 215 (CCPA 1980) (prior art suggested proportional balancing to achieve desired results in the formation of an alloy).

Applicants submit that the prior art references do not suggest purified QS21/cholesterol ratios; neither do they appreciate the advantage of the claimed ratios. The advantages of the claimed ratios have been are noted in the specification and have been subsequently supported by additional experimentation. (See Document D10 in Opposition to European Patent 0822831, provided herewith.) Applicants maintain the position that it cannot be routine to optimize a ratio that is unrecognized and the advantage of which is unknown.

Respectfully submitted,

Dated: September 8, 2008

//J. Michael Strickland //

J. Michael Strickland
Attorney for Applicant
Reg. No. 47,115
Tel. (919) 483-9024
Fax. (919) 483-7988

D10

Second Declaration of V. Henderickx

1. I, Veronique Henderickx declare that I am the same Veronique Henderickx that provided a first declaration already part of these proceedings.

2. I have been told that the Opponent (Chiron) has criticised the work that I performed, as described in my first declaration. Specifically, the Opponent stated that I had not followed the example of D1 because I stated in my declaration that:
"*The lipid film was then resuspended*" (paragraph 4, line 6)
which the Opponent said was not the same as:
"*...solution was slowly added to the dried lipid and hand shaken until the lipids were resuspended...*" as described in D1.

3. I confirm that when repeating D1 (the results of this experiment are described in my first declaration) I slowly added the solution to the dried lipid and hand shook until the lipids were resuspended exactly as described in D1. This "hand-shaking" procedure is so standard in the art of liposome production that I thought it clear from my description of the method.

4. I have been asked whether we have additional data to show that the adjuvants claimed in the patent are non-toxic, whilst similar adjuvants are toxic. I have also been asked whether sterols, other than cholesterol, also stabilise QS21 in its non-hydrolysed form and also quench its haemolytic activity in vitro. I have data to answer both of these questions in the positive. The work described was either performed by myself, or at my request.

Toxicity Study

5. This study was performed to ascertain whether the toxicity observed at a histological level with QS21 and Quil A was quenched by the addition of cholesterol. Cholesterol containing adjuvants were prepared with excess QS21 (w/w) in the form of ISCOMs, or with excess cholesterol in the form of liposomes or oil in water emulsions.

6. Groups of three rats were injected (in 50µl volumes) in the thigh muscle with one of several adjuvant or control groups (described below) and the reactogenic differences between the groups were observed.

Group	Structure	Composition	Within claim?
1	-	Phosphate Buffered Saline (PBS)	-
2	Aqueous solution	5 µg QS21	No
3	Aqueous solution	5µg Quil A	No
4	ISCOM	5 µg QS21, 1µg cholesterol, 4 µg Dioleoyl phosphatidyl choline (DOPC) 5:1	No

5:1

5	Oil in water emulsion	5 µg QS21, 5µg 3D-MPL, 1.25µl squalene, 1.25µl α-tocopherol, 0.486% TWEEN 80™	No	+ lysis see figure
6	Liposome	5 µg QS21, 25 µg cholesterol, 100 µg DOPC	Yes	1:5
7	Liposome	5 µg QS21, 5µg 3D-MPL, 25 µg cholesterol, 100 µg DOPC	Yes	1:5
8	Oil in water emulsion	10µg cholesterol, 5 µg QS21, 5µg 3D-MPL, 0.25µl squalene, 0.25µl α-tocopherol, 0.486% TWEEN 80™	Yes	1:2

7. Haemolytic activity of the groups was measured by the following technique. Red blood cells were isolated from blood and washed three times in PBS. Immediately prior to use the red blood cell pellet was dilute ten times with PBS pH 7.4. Adjuvant samples were diluted so that they contained 2.5µg of either QS21 or Quil A in a final volume of 900µl, to which 100 µl of red blood cells was added, and hand shaken. After 30 minutes at room temperature the samples were centrifuged and the Optical Density of the supernatant was measured at 540 nm. The results were as follows:

Group	Haemolytic activity (OD 540 nm)
1	0.1
2	0.652
3	0.859
4	0.854
5	Lytic (equivalent to groups 2 to 4)
6	0.097
7	0.1
8	Not lytic (equivalent to groups 1, 6 and 7)

NB, the results for groups 5 and 8 were by visual inspection only due to the high turbidity of the oil in water emulsion preventing reading of results by OD.

8. The animals were euthanased three days post injection and muscle samples were prepared for histological examination. The muscle samples were formalin fixed and cut into six slices of about 2 mm thickness. The 6 slices were dehydrated and paraffin embedded in one block. From each block, 1 section of about 7µm in thickness were stained by the Trichrome Masson method and examined microscopically.

9. Photographs of the stained slides are shown in the annexes. In Group 1, the PBS negative control, only very slight damage can be seen to the muscle tissue, often the areas of blue damage following the line of needle entry. The muscle tissue itself, however, remains alive and healthy.

10. In contrast, Groups 2 (QS21), 3 (Quil A), 4 (QS21 ISCOM), and 5 (QS21 o/w emulsion), show large areas of tissue death or necrosis at the site of injection, as shown by large grey/blue areas of indistinct dead cells.

11. There are no evident areas of necrosis to be seen in Groups 6 (QS21/cholesterol liposomes), 7 (QS21/cholesterol liposomes/3D-MPL) or 8 (QS21/cholesterol o/w emulsion) and in this respect are equivalent to the PBS negative control.

12. The samples were scored visually for the Extent, Degeneration/Necrosis, Regeneration, Inflammation of mononuclear cells and Hemorrhage (0=normal, 1=minimal, 2=slight, 3=moderate, 4=marked, 5=severe). The average results for the 6 sections are shown in the following table.

Group	Extent	Necrosis	Regeneration	Inflammation	Hemorrhage
1	1	0.2	0.7	1	0
2	3	2.7	2.2	3.7	3
3	3	2.7	1.5	3.7	2.2
4	3	3.2	1.5	3.5	2.3
5	2.8	2.2	0.2	3.8	2.0
6	1	0.2	1	1	0
7	2	0.4	0	2.3	0.2
8	1	0	0.3	1	0

in claims

13. These results show clearly that the addition of cholesterol to QS21 containing adjuvants, either in the form of an oil in water emulsion or liposome significantly reduces their haemolytic activity and in vivo toxicity. The cholesterol : QS21 ratio is however, critical to the success of this effect. Ratios where QS21 is in w/w excess does not quench the haemolytic activity or reactogenicity of QS21 at all.

Sterol stabilisation of QS21 study

14. Following the procedures set out in Example 1.4 of the Patent a number of unilamellar liposomal formulations were prepared with DOPC and cholesterol, Sitosterol, Stigmasterol or Ergosterol and mixed with QS21 at either a 2:1 or 5:1 (sterol:QS21) ratio. The samples were kept at 37°C for 16 hours, at pH 8.8, and the appearance of QS21-H, the hydrolysis degradation product of QS21, was measured by HPLC.

The results are shown in the following table:

Sample	Sterol:QS21 ratio	% QS21-H after 16 hours
QS21	-	84
QS21 + Cholesterol	2:1	10
QS21 + Cholesterol	5:1	10.2
QS21 + Sitosterol	2:1	9.6
QS21 + Sitosterol	5:1	8.7
QS21 +	2:1	20.7

31-05-02

Stigmasterol		
QS21 + Stigmasterol	5:1	16.3
QS21 + Ergosterol	2:1	35.8
QS21 + Ergosterol	5:1	21.2

15. All of the sterols tested significantly stabilised QS21 from hydrolysis.

16. In my experience, all sterols that I have tested in the past, including cholesterol, sitosterol, stigmasterol, ergosterol and ergocalciferol, quench the lytic activity of QS21.

Conclusion

17. The adjuvants described in the patent contain QS21 which is stabilised and detoxified by sterols at a ratio of 1:1 to 1:100 w/w (QS21:sterol). The adjuvants falling within these ratios are not toxic as shown in the rat muscle necrosis studies, and these same adjuvants also have quenched haemolytic activity. The QS21 in these adjuvants is also stabilised in its adjuvant active form. QS21 containing adjuvants with excess QS21 (outside of the claimed range) are toxic, haemolytic, and in my opinion the QS21 would not be stable.

18. In this and other experiences, ratios of 1:1 and excess sterol quench the toxicity of QS21 and stabilise the QS21 in its non-hydrolysed form. I have no reason whatsoever to believe that if you increase the ratio of Cholesterol:QS21 to closer to 100:1 (w/w) that these adjuvants would not have the same non-toxic and stable characteristics as those shown in the Patent and in this declaration.

I declare the above to be true to the best of my knowledge and belief.

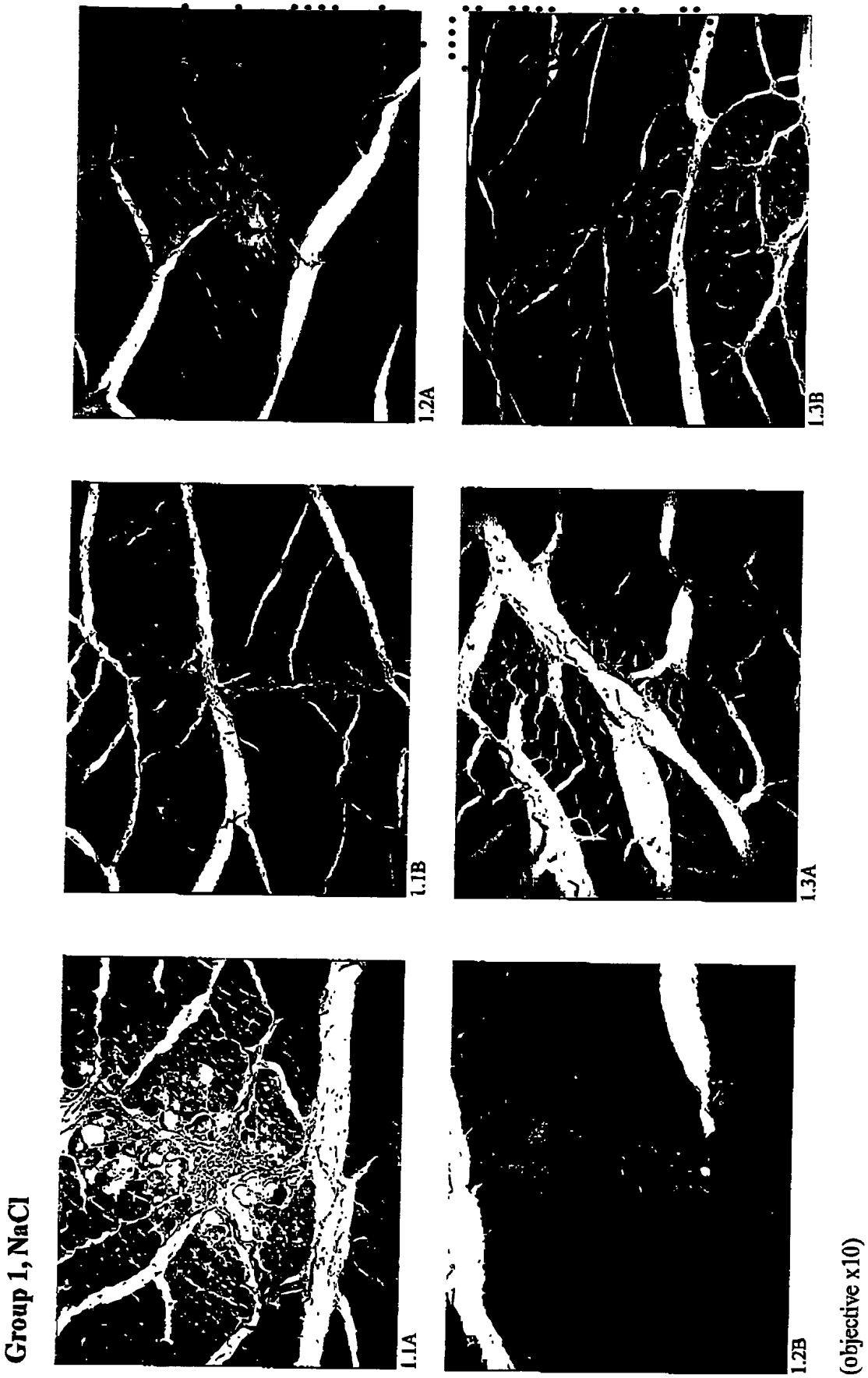
Hendericks

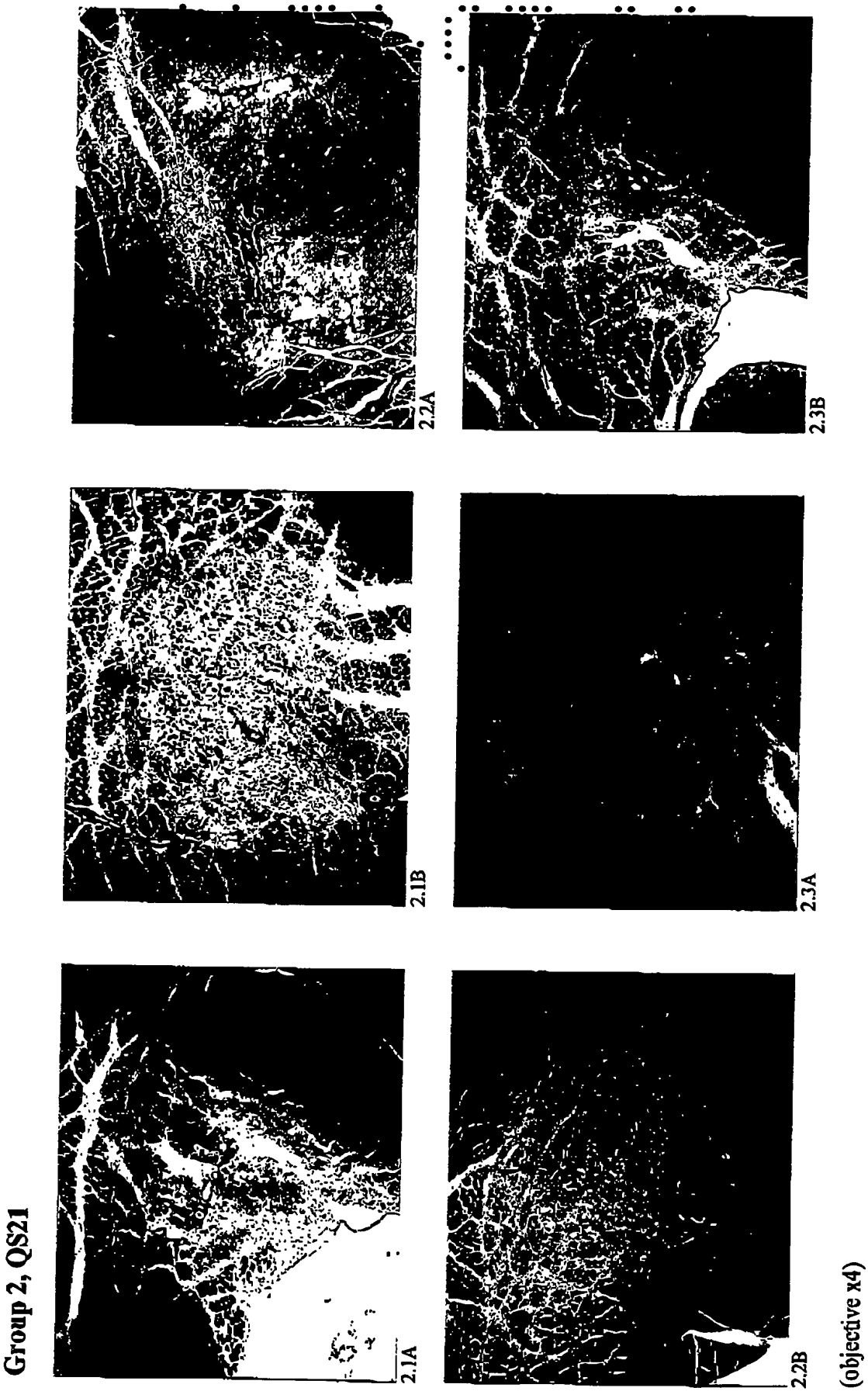
.....

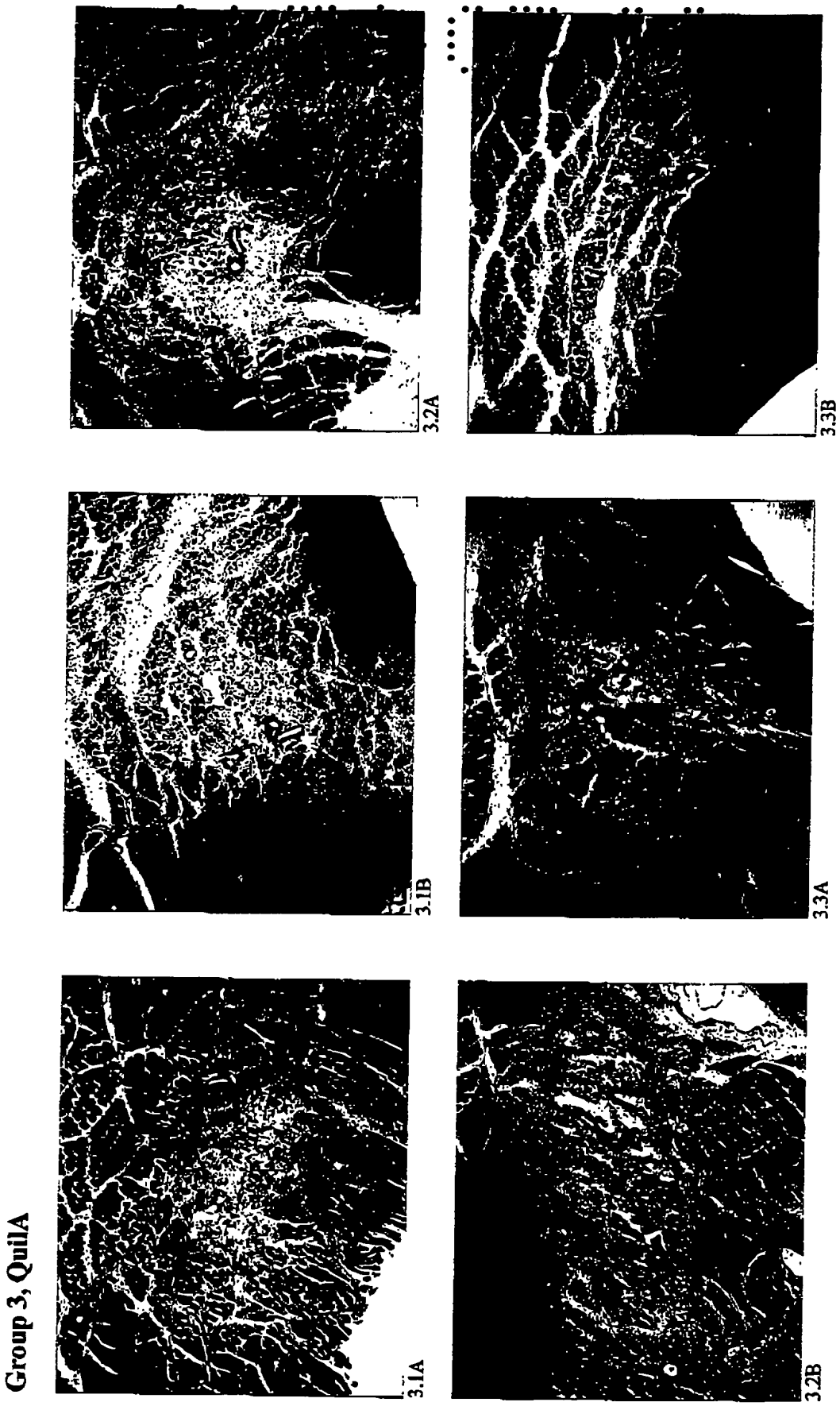
Veronique Henderickx

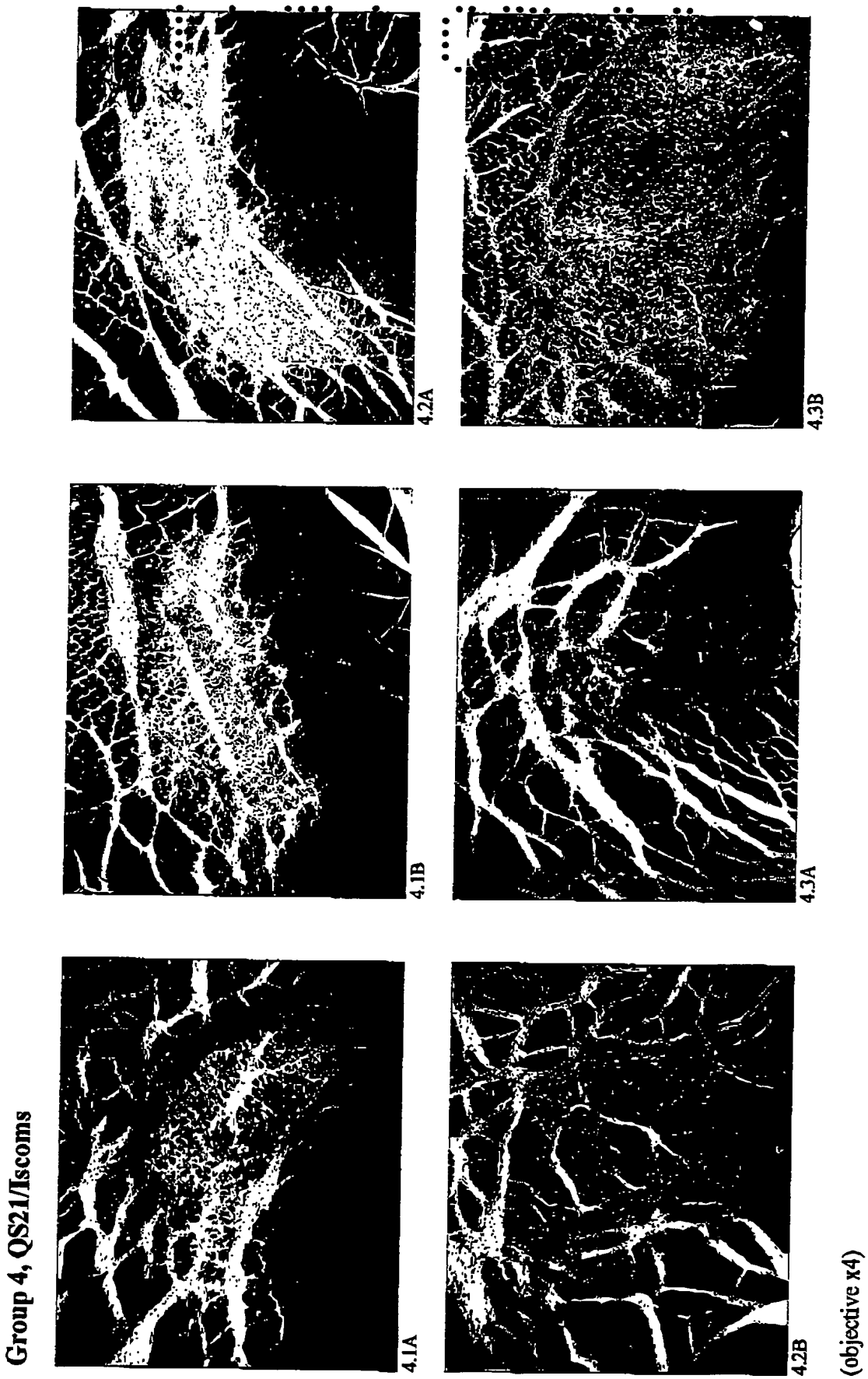
30.05.2002

Date


Group 1, NaCl
1.1A
1.1B
1.2A
1.2B
1.3A
1.3B
(objective x10)

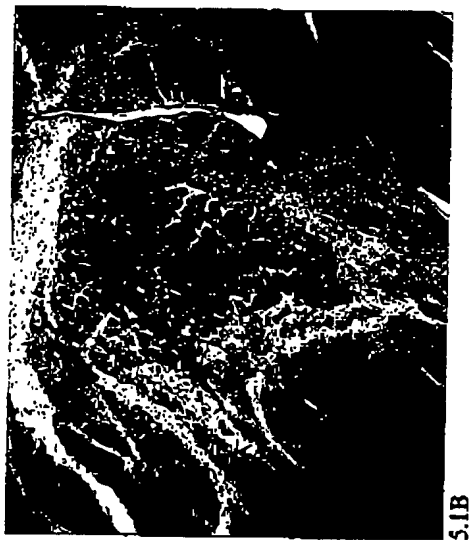

Group 2, QS21
2.1A
2.1B
2.2A
2.2B
2.3A
2.3B
(objective x4)


Group 3, QuilA
3.1A
3.1B
3.2A
3.2B
3.3A
3.3B
(objective x4)


Group 4, QS21/Iscoms
4.1A
4.1B
4.2A
4.2B
4.3A
4.3B
(objective x4)

Group 5, oil in water emulsion QS21/MPL


5.1A


5.1B

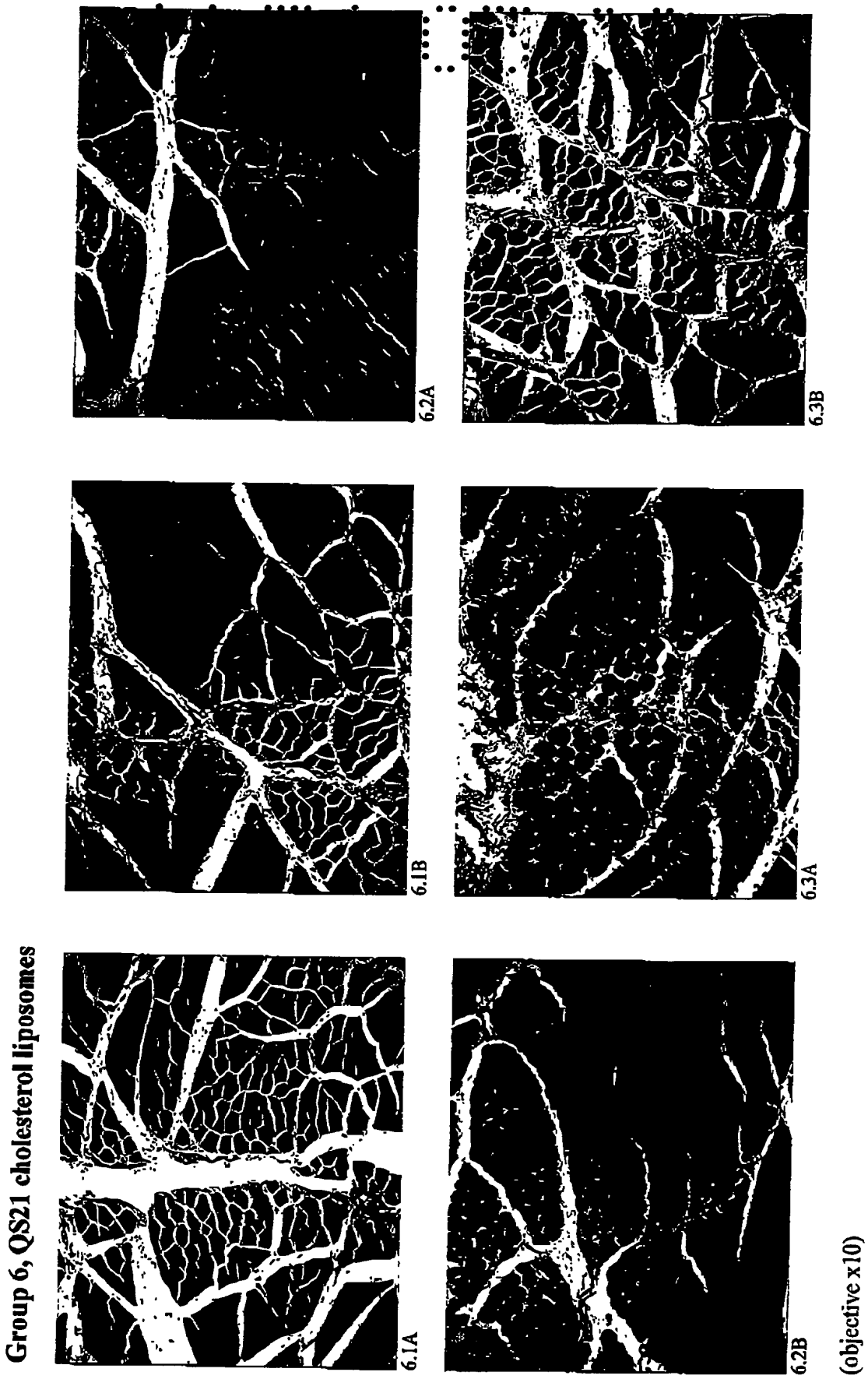

5.2B

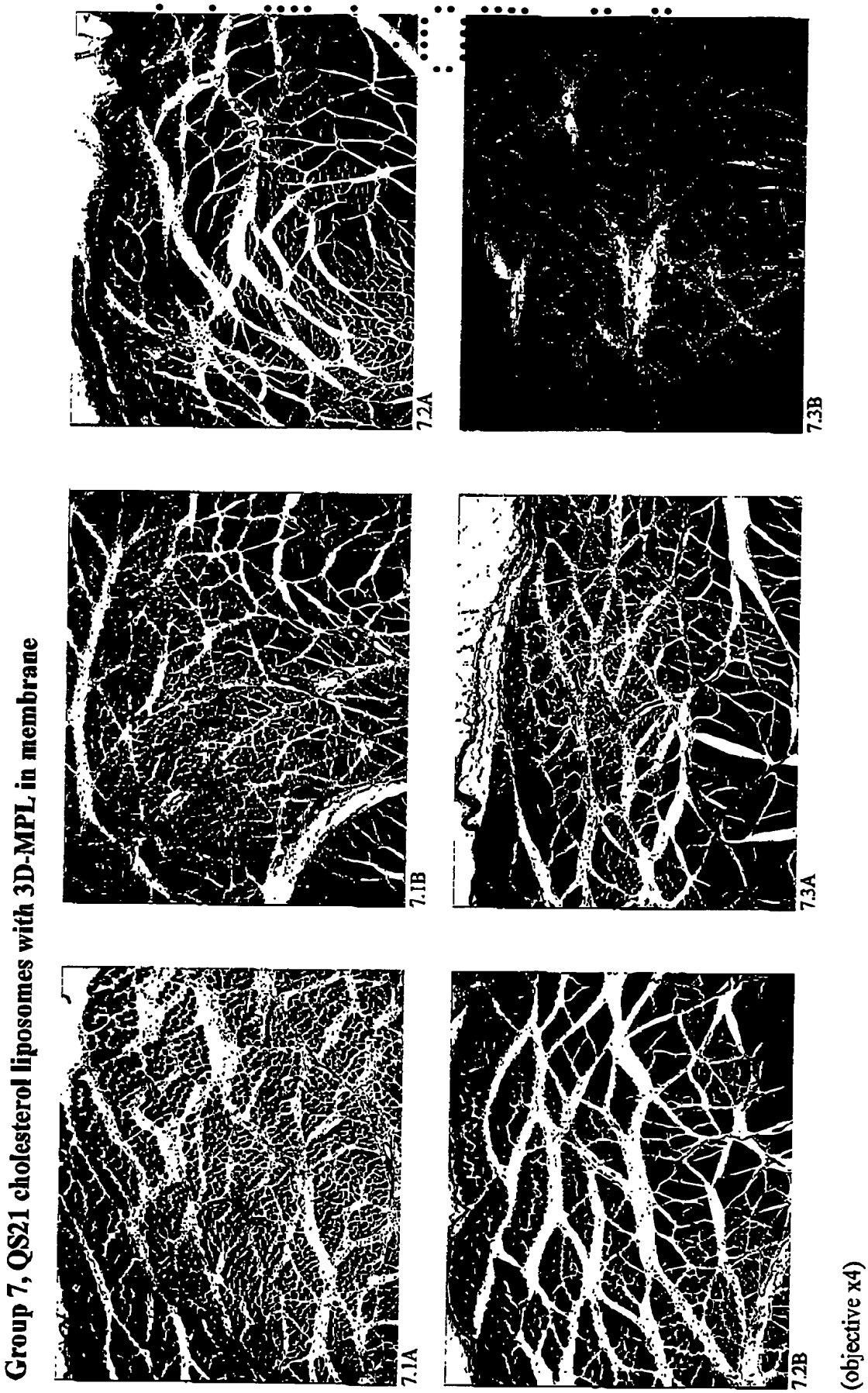

5.2A


5.3A


5.3B

(objective x4)


Group 6, QS21 cholesterol liposomes
6.1A
6.1B
6.2A
6.2B
6.3A
6.3B
(objective x10)

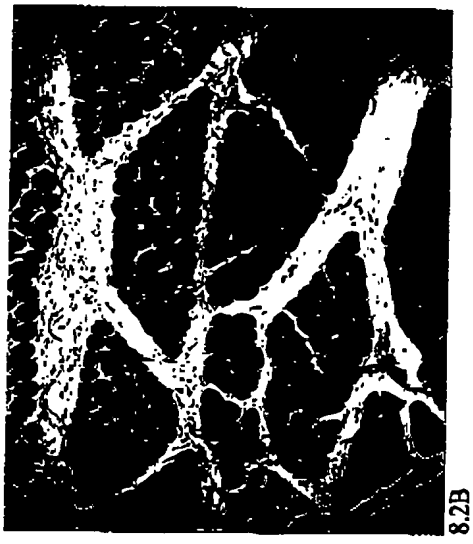

Group 7, QS21 cholesterol liposomes with 3D-MPL in membrane
7.1A
7.1B
7.2A
7.2B
7.3A
7.3B
(objective x4)

Group 8, oil in water emulsion cholesterol QS21/MPL

8.1A

8.1B

8.2A



8.2B

8.3A

8.3B

(objective x10)